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Protective Effects of Dietary Safflower (*Carthamus tinctorius*) on Experimental Coccidiosis

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This study was conducted to evaluate the effects of dietary safflower leaf on protective immunity against coccidiosis, the most economically important parasitic disease of poultry. White Leghorn chickens were fed a standard diet with or without safflower leaf and were either uninfected or orally infected with 5,000 sporulated oocysts of *Eimeria acervulina*. Protective immunity was assessed by body weight gain, fecal oocyst shedding, splenic lymphocytes proliferation, T lymphocyte subpopulations, and proinflammatory cytokine gene expression. We observed that the effect of safflower on experimental coccidiosis was dependant on the dose of the supplement used. A 0.1% (wt/wt) safflower-supplemented diet increased body weight gains of coccidia-infected chickens to a level identical to that of uninfected controls, and significantly reduced fecal oocyst shedding compared with animals that were given a non-supplemented standard diet. Furthermore, increased splenic lymphocyte proliferation as well as greater percentages of $CD4^+$ T cells and decreased $CD8^+$ cells were observed in animals fed a 0.1% safflower-supplemented diet. Finally, IFN-γ, IL-8, IL-15 and IL-17 transcripts in the 0.1% safflower-supplemented group were higher than the non-supplemented controls. These results indicate that safflower leaf when given as a dietary supplement possesses immune-enhancing properties that augment protective immunity against experimental coccidiosis.

Key words: coccidiosis, cytokines, immunity, safflower

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Introduction

Coccidiosis is caused by several species of *Eimeria* and is an important disease in poultry production being responsible for annual economic losses estimated to be $3 billion (Lillehoj *et al.,* 2004). Avian coccidiosis has traditionally been controlled by chemoprophylaxis using anticoccidial synthetic products or antibiotic ionophores. However, with increasing concerns over the emergence of drug-resistant *Eimeria* strains, alternative control methods are needed. Recent studies from our laboratory have demonstrated that dietary supplementation with *Pediococcus*-based probiotics enhanced immunity against *Eimeria acervulina* (Lee *et al.,* 2007a; Lee *et al.,* 2007b). In addition, feeding a *Fomitella fraxinea*-derived lectin or methanol extracts of traditional medical fruits, such as the Oriental plum (*Prunus salicina*), also were effective in enhancing immune resistance to experimental *Eimeria* infection (Dalloul *et al.,* 2006; Lee *et al.,* 2008b). Mechanistically, the effects of natural food and herbal products on host defense against microbial infections and tumors have shown a good correlation with their ability to enhance various *in vitro* correlates of immunity, for example lymphocyte proliferation (Kim *et al.,* 2004; Lee *et al.,* 2005; Pandey *et al.,* 2005; Park *et al.,* 2004).

Safflower (*Carthamus tinctorius*), which belongs to the Compositae family, has been cultivated for more than two thousand years and has historically been used as a herbal medicine against infectious diseases and cancers. Due to renewed interests in the use of natural products to enhance human and animal health, safflower products have received much attention as immunomodulating agents (Lee *et al.,* 2007c; Lee *et al.,* 2008a). In animals, safflower showed no toxicity as a novel pasture species for dairy sheep or late-pregnancy dairy cows (Landau *et al.,* 2004;

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Landau *et al.,* 2005). Safflower seed oil inhibited the production of proinflammatory cytokines by endotoxin (LPS)-stimulated human monocytes (Takii *et al.,* 2003) and safflower petals contain polysaccharides that activated macrophages *in vitro* (Ando *et al.,* 2002). However, few studies have reported the effects of safflower on immunity against a specific pathogenic microorganism. Therefore, we conducted the current investigation to examine the effect of the dietary safflower leaf on protective immunity against experimental coccidiosis in chickens.

Materials and Methods

Experimental animals, diets, and coccidia infection

All experiments were performed according to the guidelines established by the Beltsville Area Institutional Animal Care and Use Committee. Fertilized eggs of specific pathogen-free White Leghorn chickens were obtained from SPAFAS (Charles River Laboratories, Preston, CT) and were hatched at the Animal and Natural Resources Institute, USDA (Beltsville, MD). One-day-old male chicks ($n=40$) were randomly assigned to 4 groups and fed a standard chicken diet either with safflower leaves, supplemented at 0.1% (w/w) (SF 0.1) or 0.5% (SF 0.5) of the diet (10 birds each), or without safflower leaves, (20 birds; 10 uninfected controls [control] and 10 infected controls [SF 0]), *ad libitum* for 3 weeks. Safflower leaf diets were prepared by mixing the standard chicken diet and freeze-dried safflower leaf powder supplied by the National Rural Resources Development Institute (Suwon, South Korea). All diets were formulated to meet the nutrient requirements for chickens as recommended (National Research Council, 1994). Thirty birds (N=10/group; SF 0, SF 0.1 and SF 0.5) were orally inoculated with 5,000 sporulated oocysts of *Eimeria acervulina* on day 12 post-hatch as described (Min *et al.,* 2001), while the 10 remaining control birds were uninfected.

Measurement of body weight and oocyst shedding

Body weight gains were calculated between 0 and 10 days post-inoculation (dpi) as described (Lee *et al.,* 2007 a). Oocyst shedding was assessed as described (Lee *et al.,* 2007a). Briefly, fecal droppings were collected daily between 5 and 10 dpi and pooled fecal material was suspended in 3 L of water. Two 35 mL samples were taken, diluted, and the number of oocysts was counted microscopically using a McMaster chamber. The total number of oocysts was calculated using the formula: total oocysts=oocyst count×dilution factor×(fecal sample volume/counting chamber volume).

Splenic lymphocytes proliferation

Spleens were removed at 10 dpi and placed in a Petri dish with 10 mL of Hank's balanced salt solution (HBSS) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO). Single cell suspensions of splenic lymphocytes were prepared (Kaspers *et al.,* 1994) and proliferation was determined as described (Okamura *et al.,* 2004). In brief, splenic lymphocytes obtained by Ficoll density gradient centrifugation were washed three times with PBS and adjusted to 1×10^7 cells/mL in enriched RPMI-1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin. Splenic lymphocytes (100 μL/well) were cultured in 96-well flat bottom plates and incubated at 41℃ in a humidified incubator (Forma, Marietta, OH) with 5% CO_2 for 48 h. Cell proliferation was determined with 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt (WST-8, Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) as described (Miyamoto *et al.,* 2002). Optical density (OD) was measured at 450 nm using a microplate reader (BioRad, Richmond, CA).

Flow cytometric analysis

Indirect immunofluorescence staining and flow cytometric analysis of splenic lymphocytes were performed as described previously (Lillehoj, 1994). Singe cell suspensions of fresh cells were resuspended in 1.0 mL of flow cytometer buffer (HBSS containing 3% FBS and 0.01% sodium azide). One hundred μL aliquots of cell suspensions (approximately 10^6 cells) were incubated on ice for 40 min with 100 μL of appropriately diluted monoclonal antibody (mAb) against the surface markers CD4, CD8, αβ-T cell receptor (αβ-TCR) or γδ-TCR as described (Hong *et al.,* 2006a). After washing twice with 2.0 mL of flow buffer, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma) for 30 min on ice, washed twice, resuspended in 2.0 mL, and analyzed with an Epics model XL flow cytometer (Coulter, Miami, FL). Data were obtained from a total of 10^4 viable cells.

Quantification of cytokine and chemokine mRNA levels

Cytokine and chemokine gene expression analysis was carried out using real-time RT-PCR as described (Hong *et al.,* 2006a; Hong *et al.,* 2006b). At 10 dpi, the intestinal duodenum was removed, cut longitudinally, and washed three times with ice-cold HBSS containing 100 U/mL of penicillin and 100 μg/mL of streptomycin. The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM EDTA and 5% FBS and incubated for 20 min at 37℃ with constant swirling. Cells released into the supernatant were washed twice with HBSS and filtered by a syringe containing nylon wool. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μL of 10X reaction buffer (Sigma), incubated for 15 minutes at room temperature, 1.0 μL of stop solution was added to inactivate DNase I, and the mixture was heated at 70℃ for 10 minutes. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, 5.0 μg of RNA was combined with 10X the first strand buffer, 1.0 μL of oligo (dT) primer (5.0 μg/μL), 0.8 μL of dNTP mix (25

Table 1 **Oligonucleotide primers used in this study**

RNA target	Primer sequences	PCR product size (bp)	Accession no.
GAPDH			
Forward	5′-GGTGGTGCTAAGCGTGTTAT-3′	264	K01458[1)]
Reverse	5′-ACCTCTGTCATCTCTCCACA-3′		
IFN-γ			
Forward	5′-AGCTGACGGTGGACCTATTATT-3′	259	Y07922[2)]
Reverse	5′-GGCTTTGCGCTGGATTC-3′		
IL-8			
Forward	5′-GGCTTGCTAGGGGAAATGA-3′	200	AJ009800[3)]
Reverse	5′-AGCTGACTCTGACTAGGAAACTGT-3′		
IL-15			
Forward	5′-TCTGTTCTTCTGTTCTGAGTGATG-3′	243	AF139097[4)]
Reverse	5′-AGTGATTTGCTTCTGTCTTTGGTA-3′		
IL-17			
Forward	5′-CTCCGATCCCTTATTCTCCTC-3′	292	AJ493595[5)]
Reverse	5′-AAGCGGTTGTGGTCCTCAT-3′		

[1)] Panabieres *et al.,* 1984.
[2)] Kaiser *et al.,* 1998.
[3)] Kaiser *et al.,* 1999.
[4)] Lillehoj *et al.,* 2001.
[5)] Lillehoj and Min, 2002.

mM of each dNTP), and RNase-free water to a total volume of 19 μL. The mixture was incubated at 65℃ for 5 min, cooled to room temperature, 50 U of StrataScript reverse transcriptase was added, the mixture was incubated at 42℃ for 1 hr, and the reaction was stopped by heating at 70℃ for 5 min. Quantitative RT-PCR oligonucleotide primers for chicken cytokines along with the GAPDH internal control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from duodenal lymphocytes using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA. Levels of individual transcripts were normalized to those of GAPDH analyzed by the Q-gene program (Muller *et al.,* 2002). Each analysis was performed in triplicate. To normalize individual replicates, the logarithmic-scaled raw data unit Cycle Threshold (CT) was transformed into linear unit of normalized expressions and calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression (MNE) using the Q-gene program (Hong *et al.,* 2006a; Hong *et al.,* 2006b; Hong *et al.,* 2006c; Hong *et al.,* 2006d; Muller *et al.,* 2002).

Statistical analyses

Statistical analyses were performed using SPSS 12.0K software for Windows. All data was expressed as means± SEM. The ANOVA test was used to test for differences between treatment groups. Duncan's multiple range test was used to analyze significant differences among the means at $P<0.05$.

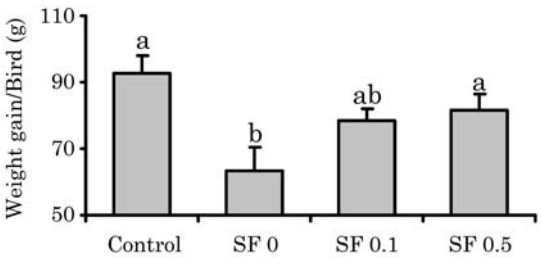

Fig. 1. **Body weight gains of White Leghorn chickens fed safflower leaf-supplemented diets.** Chickens were fed from hatch with diets supplemented with 0% (control, SF 0), 0.1% (SF 0.1) or 0.5% (SF 0.5) safflower leaf, animals were uninfected (control) or infected with 5,000 *E. acervulina* oocysts at 12 days post-hatch, and body weights were measured at 0 and 10 dpi. Each value represents the mean±SEM values from 10 chickens. Bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Duncan's multiple range test.

Results

Body weight gain during experimental coccidiosis

Mean body weight gains (g) of uninfected control and *E. acervulina*-infected groups on a normal diet (SF 0) and on safflower leaf-supplemented diets (SF 0.1, SF 0.5) were calculated over the 10 day infection period. As shown in Fig. 1, weight gain was significantly reduced in the *E. acervulina*-infected SF 0 group compared with uninfected birds. In contrast, safflower leaf-supplemented, parasite-infected chickens exhibited body weight gains identical to

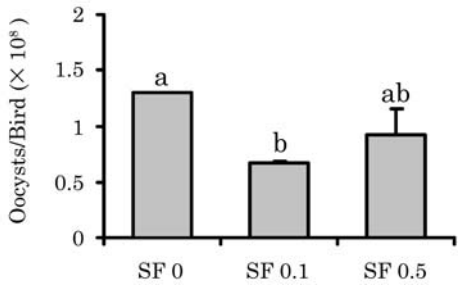

Fig. 2. **Fecal oocyst shedding of White Leghorn chickens fed safflower leaf-supplemented diets.** Chickens were fed from hatch with diets supplemented with 0% (SF 0), 0.1% (SF 0.1) or 0.5% (SF 0.5) safflower leaf, animals were infected with 5,000 *E. acervulina* oocysts at 12 days post-hatch, and fecal oocysts were enumerated at 5−10 dpi. Each value represents the mean±SEM values from 10 chickens. Bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Duncan's multiple range test.

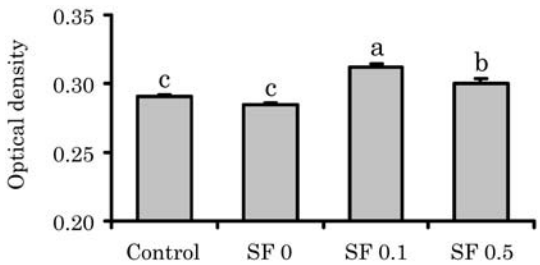

Fig. 3. **Spleen lymphocyte proliferation of White Leghorn chickens fed safflower leaf-supplemented diets.** Chickens were fed from hatch with diets supplemented with 0% (control, SF 0), 0.1% (SF 0.1) or 0.5% (SF 0.5) safflower leaf, animals were uninfected (control) or infected with 5,000 *E. acervulina* oocysts at 12 days post-hatch, and splenocyte proliferation was determined at 10 dpi. Each value represents the mean±SEM values from 10 chickens. Bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Duncan's multiple range test.

that of uninfected controls.

Oocyst shedding during experimental coccidiosis

Fig. 2 shows that oocyst shedding by the *E. acervulina*-infected and safflower leaf-supplemented SF 0.1 group was significantly decreased by 48.6% compared with the SF 0 group. No oocysts were shed by uninfected chickens (data not shown).

Splenic lymphocytes proliferation

As shown in Fig. 3, splenic lymphocytes proliferation was significantly increased in the safflower leaf-supplemented groups SF 0.1 and SF 0.5 compared with the SF 0 control group. Interestingly, although there was no significant difference in splenocytes proliferation between the uninfected control and *E. acervulina*-infected SF 0 groups, the infected SF 0.1 group exhibited significantly increased proliferation compared with the infected SF 0.5 group.

Lymphocyte subpopulations

The percentages of $CD4^+$ cells increased in all *E. acervulina*-infected groups compared with the uninfected control group, but this difference was significant only with the SF 0.1 group (Table 2). In contrast, the percentages of $CD8^+$ cells were significantly decreased in the SF 0.1 and SF 0.5 groups compared with the uninfected control and *E. acervulina*-infected SF 0 groups. Finally, while there were no changes in the percentages of $\alpha\beta$-TCR^+ cells in any of the experimental groups, $\gamma\delta$-TCR^+ cells were significantly higher in the SF 0 group than the uninfected control group. However, this difference from the uninfected control group was not seen in the SF 0.1 or SF 0.5 animals.

Cytokines and chemokine transcript levels

Duodenal lymphocyte IFN-γ transcript levels were significantly higher in the SF 0.1 and SF 0.5 groups compared with the uninfected control and SF 0 groups (30- and 27-fold increases respectively compared with the SF 0 group) (Fig. 4). Similarly, transcript levels for IL-8, IL-15 and IL-17 in SF 0.1 animals were significantly increased compared with the SF 0 birds (2.4-, 3.4- and 6.2-fold, respectively).

Table 2. **Splenic lymphocytes subpopulation (%) of White Leghorn chickens fed safflower leaf-supplemented diets**

Group	Diet	$CD4^+$	$CD8^+$	$\alpha\beta$-TCR^+	$\gamma\delta$-TCR^+
Uninfected	Control	46.6^b	47.2^a	67.3	7.5^{bc}
Infected	SF 0	51.2^b	46.4^a	65.8	11.6^a
	SF 0.1	61.9^a	29.5^b	62.3	9.8^{ab}
	SF 0.5	50.3^b	27.0^b	59.7	6.7^c

Chickens were fed from hatch with diets supplemented with 0% (control, SF 0), 0.1% (SF 0.1) or 0.5% (SF 0.5) safflower leaf and animals were uninfected (control) or infected with 5,000 *E. acervulina* oocysts at 12 days post-hatch. Spleen lymphocytes were analyzed for expression of the indicated cell surface markers by flow cytometry as described in the Materials and Methods. Data are expressed as mean percentages of splenic lymphocytes at 10 dpi. Values in the same column with different superscript are significantly different ($P<0.05$).

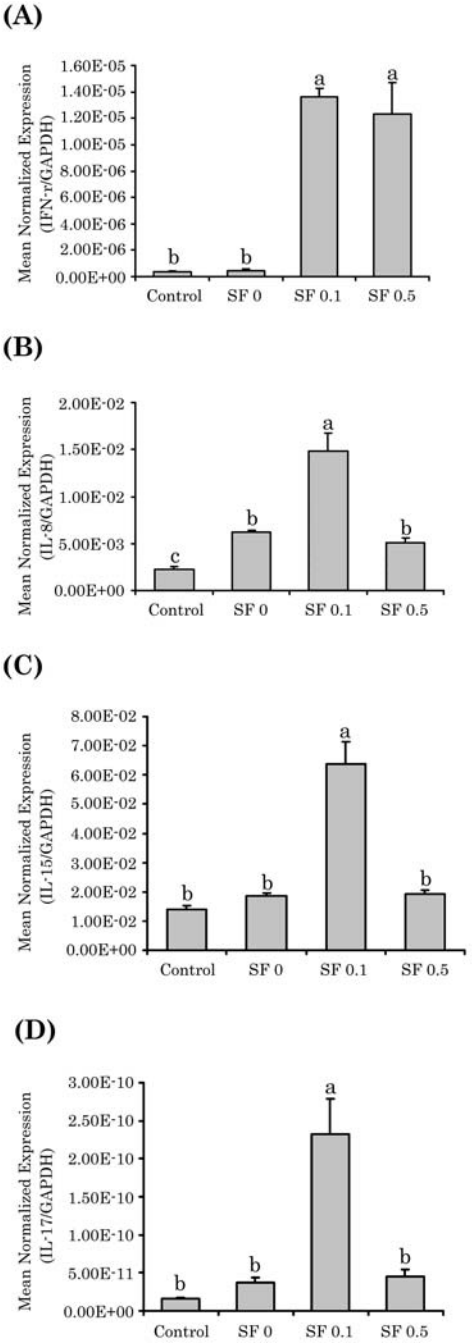

Fig. 4. **Cytokines and chemokine mRNA levels in duodenal lymphocytes of White Leghorn chickens fed safflower leaf-supplemented diets.** Chickens were fed from hatch with diets supplemented with 0% (control, SF 0), 0.1% (SF 0.1) or 0.5% (SF 0.5) safflower leaf, animals were uninfected (control) or infected with 5,000 *E. acervulina* oocysts at 12 days post-hatch, duodenal lymphocytes were isolated at 10 dpi, and levels of transcripts encoding IFN-γ (A), IL-8 (B), IL-15 (C) and IL-17 (D) were quantified by real-time RT-PCR and normalized to GAPDH mRNA. Each value represents the mean$\pm$SEM values from 10 chickens. Bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Duncan's multiple range test.

Discussion

E. acervulina infects the intestinal duodenum causing a multitude of symptoms, including diarrhea, body weight loss, and occasional mortalities in younger chickens (Williams, 2002; Yunus *et al.,* 2005). Historically, the severity of *Eimeria* infection has been assessed by reduced body weight gain and the excretion of fecal oocysts (Idris *et al.,* 1997). In this study, significantly decreased oocyst shedding, but not increased body weight gain, was observed in the SF 0.1 group compared with the SF 0 group, suggesting that the immunomodulatory effect of safflower may independently influence these two parameters of infection. These results are in agreement with other reports indicating that there is no direct correlation between the *Eimeria*-induced reduced body weight gain and fecal oocyst shedding during experimental coccidiosis (Lee *et al.,* 2007a; Lillehoj and Okamura, 2003).

Only the SF 0.1 group showed enhanced splenocyte proliferation and an increased percentage of $CD4^+$ spleen lymphocytes compared with the SF 0 group. In contrast, the percentages of $CD8^+$ cells were significantly lower in the SF 0.1 and SF 0.5 groups than the *E. acervulina*-infected SF 0 control group. These results are similar to the previous study demonstrating that treatment of mice with oat β-glucan decreased the percentage of $CD8^+$ cells and increased $CD4^+$ cells, concomitant with enhanced disease resistance against *Staphylococcus aureus* or *E. vermiformis* infections (Yun *et al.,* 2003). The increased $CD4^+$ and decreased $CD8^+$ cell population described in this study suggests a protective function of these cells in innate immune response against *E. acervulina* infection. $CD4^+$ cells are the major cells producing the proinflammatory cytokine IFN-γ in response to antigen challenge (McSorley *et al.,* 2000) and IFN-γ plays an important role in protective immunity to experimental coccidiosis (Lillehoj and Choi, 1998). Unlike cells expressing the CD 4, CD8 or $\alpha\beta$-TCR surface markers, and as reported by others (Hong *et al.,* 2006a; Bessay *et al.,* 1996), $\gamma\delta$-TCR^+ cells were increased in SF 0 chickens compared with uninfected controls. Thus, the effect of safflower was to hinder the increase in $\gamma\delta$-TCR^+ cells induced by coccidia infection. While previous studies suggested a role for $\gamma\delta$-TCR^+ cells in mediating a cytotoxic effect against *Eimeria* parasites (Lillehoj, 1989), our current results appear to be independent of the observation that treatment of chickens with dexamethasone significantly decreased the percentage of $CD8^+$ and $\gamma\delta$-TCR^+ cells, and increased $CD4^+$ cells (Isobe and Lillehoj, 1992).

Host immunity to *Eimeria* infection is accompanied by the activation of a series of cell-mediated immune responses and the production of cytokines involved in local gut immunity including IFN-γ, IL-8, IL-15 and IL-17 (Hong *et al.,* 2008; Lee *et al.,* 2007c, 2008b; Lillehoj *et al.,* 2001, 2002). IFN-γ is a common marker of cellular immunity where higher levels are associated with protective immune responses to coccidia infections (Lillehoj,

1996; Choi *et al.,* 1999; Min *et al.,* 2003; Lillehoj *et al.,* 2004; Hong *et al.,* 2006b). For example, administration of recombinant IFN-γ to chickens significantly hindered the intracellular development of *Eimeria* parasites (Lillehoj *et al.,* 1998). In the current study, IFN-γ gene expression was significantly up-regulated in chickens fed 0.1% and 0.5% safflower leaf, indicating that this natural product may exert its protective effect through the enhancement of IFN-γ production. Chickens fed the 0.1% safflower-supplemented diet also showed greater levels of transcript encoding IL-8, IL-15 and IL-17 compared with the SF 0 group. IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells, and is one of the major mediators of the inflammatory response. Its primary function is to recruit neutrophils to sites of infection to phagocytose invading pathogens (Utgaard *et al.,* 1998; Wolff *et al.,* 2008). IL-15 is a cytokine that stimulates the proliferation of chicken T lymphocytes and NK cells (Choi and Lillehoj, 2000; Lillehoj *et al.,* 2001) while IL-17 induces the production of other cytokines such as IL-1β, IL-6, IL-8, G-CSF, GM-CSF, TNF-α and TGF-β (Hong *et al.,* 2008; Veldhoen *et al.,* 2006). IL-15 enhanced protective immunity to coccidiosis when co-administered with an experimental DNA vaccine encoding the *Eimeria* 3-1E (profilin) gene (Min *et al.,* 2001). Chickens vaccinated with 3-1E DNA in combination with IL-8 or IL-15 shed significantly fewer fecal oocysts compared with chickens vaccinated with 3-1E alone. Additionally, *in ovo* co-vaccination with 3-1E plus IL-15 or IL-17 reduced oocyst output beyond that diminished by 3-1E alone (Lillehoj *et al.,* 2001). Thus, we propose that feeding safflower-supplemented diets to chickens enhances protective immunity against coccidiosis by stimulating local lymphocyte proliferation and cytokine production. Interestingly, this protective effect was highly dose-dependent since the SF 0.1 group showed greater activation of cell-mediated immunity and reduced fecal oocyst shedding compared with the SF 0.5 group. The addition of safflower leaves to chicken feed at the appropriate concentrations may therefore provide an alternative method against coccidiosis, particularly if and when future restrictions are placed on the use of anti-coccidial drugs in commercial production settings.

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